Developmental Expression of The Rat Rod Photoreceptor cGMP-Gated Cation Channel

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Abstract

DEVELOPMENTAL EXPRESSION OF THE RAT ROD PHOTORECPTOR cGMP-GATED CATION CHANNEL.

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The appearance of cGMP-gated cation channel protein in the postnatal rat retina has been studied by fluorescence immunocytochemistry of radial retinal sections and immunoblots of retinal membrane proteins. Channel immunoreactivity was first detectable with RCNGC1-7H2 monoclonal antibody at postnatal day 7 (PN7) by both methods. Immunocytochemical label in retinal sections was localized to the outer segments, and immunoreactivity increased with increasing age. We also compared the developmental appearance of the cGMP-gated cation channel to that of other phototransduction proteins and developmental markers. RET-P2, a monoclonal antibody recognizing the 39kDa rds/peripherin disc protein, first labeled outer segments at PN7, coincident with cGMP-gated cation channel expression. Double labeling of the same section of PN7 rat retina with RET-P2 and R309 (a polyclonal antiserum against the rod cGMP-gated cation channel) revealed identical patterns of labeling. Similarly, double-labeling with RCNGC1-7H2 and an antibody against the rod cGMP-phosphodiesterase gave coincident labeling, suggesting co-ordinate expression mechanisms of phototransduction proteins with each other and with outer segment structural proteins.

Acknowledgments

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Introduction

The terminal differentiation of rod photoreceptors is accompanied by the expression of a variety of cell type-specific molecules, including proteins involved in the visual transduction cascade. In rodents, the appearance of these molecules begins shortly after birth when the majority of rod precursors enter their final mitosis (Young, 1984; Carter-Dawson and LaVail, 1979). The differentiation of rods is thought to be complete by postnatal day 12 (PN12) since at this stage a normal electroretinogram (ERG) can be recorded (Weidman and Kuwabara, 1968, 1969). Since light responses can be detected earlier, both electrophysiologically and biochemically, it is likely that many of the components of the transduction cascade are expressed earlier, probably by the end of the first postnatal week (Ratto et al., 1991; Hicks and Barnstable, 1987a).

Previous studies have shown that the first rod photoreceptor-specific molecule to appear during retinal development is the visual pigment protein opsin. In central rat retina opsin can be detected immunocytochemically in a few cells just before birth (Hicks and Barnstable, 1987b). Opsin expression increases in a center to periphery gradient such that by PN5 all rods are opsin positive. Expression of opsin is under transcriptional control and its pattern essentially follows that of rod generation (Treisman et al., 1988; Hicks and Barnstable, 1987b). In culture, immunoreactive opsin protein is detectable in individual cells approximately 48 hours after their final mitosis, although *in vivo* the relationship between cell birth and opsin expression appears to be more complex (Watanabe and Raff, 1990; Cepko, 1996). Other studies have detected expression of components of the visual transduction cascade, including transducin, rhodopsin kinase and cGMP-phosphodiesterase (cGMP-PDE), in the second part of the first postnatal week, a stage that coincides with the onset of outer segment formation and the formation of the outer plexiform layer (Erdos et al., 1989; Ho et al., 1986; Colombaioni and Strettoi, 1993; Weidman and Kuwabara, 1968).

Light responses are converted into biochemical changes through a transduction cascade that results in changes in the concentration of cGMP (Stryer, 1986). This in turn leads to changes in rod membrane potential by altering the opening of a cyclic nucleotide-gated (CNG) channel (Fesenko et al., 1985; Kaupp et al., 1989; Yau and Baylor, 1989). Although RNA expression for this channel has been measured during rat retinal development, interpretation of these results is difficult because the channel is expressed by other cells, including retinal ganglion cells and Müller cells (Ahmad et al., 1990; 1994; Kusaka et al., 1996).

Thus, it is unclear whether the rod CNG channel is expressed at the same time as other components of the transduction cascade or whether it is expressed later and serves as a rate-limiting step for the detection of the transduction cascade by electrical recording.

Statement of Purpose

In this study we have addressed some of these questions using a series of antibodies that recognize rod-specific molecules. In particular we have used monoclonal (RCNGC1-7H2) and polyclonal (R309) antibodies raised against a C-terminal peptide of the α-subunit of the rat rod CNG channel (Barnstable & Wei, 1995), and have compared channel expression with that of other rod-specific molecules. We have found that rod CNG channel expression occurs at the same time as that of other transduction molecules and that outer segment-specific molecules seem to be localized to this structure from the time of their earliest detectable expression. This finding is of importance because it is primarily the activity of the CNG channel that regulates the membrane potential of the rod and thus can affect release of neurotransmitter. Glutamate release by the rods could influence synapse formation in both the outer and inner plexiform layers which occurs over the same time period as outer segment formation (Hendrickson, 1996). Finally, our results argue for the co-ordinate regulation of visual transduction cascade components and outer segment structural proteins.

Methods

All procedures and experiments listed below were performed by the MD candidate. Generation of antibodies and reagents were performed by laboratory technician.

Antibodies. All antibodies used in this study have been described previously and their specificities are listed in Table 1 below. All antibodies were titred and were used at the highest dilutions found to give maximal labeling.

Table 1. *Antibodies used in this study*

Antibody	Type	Specificity	Reference
RCNGC1-7H2	Mono	rod cGMP-gated cation channel (alpha subunit)	Barnstable and Wei, 1995
RET-P2	Mono	rod outer segments	Barnstable, 1980
PDE-4	Poly	cGMP phosphodiesterase	Erdos et al., 1989
R309	Poly	rod cGMP-gated cation channel (alpha subunit)	Barnstable and Wei, 1995

Immunocytochemistry. All studies were carried out using Long-Evans rats and animals were staged such that the day of birth is defined as PN1. Eyes from neonatal and adult rats were fixed by immersion in 4% paraformaldehyde in PBS following CO_2 inhalation and rapid dissection. Tissues were cryoprotected in 30% sucrose and frozen in OCT compound (Miles, Elkhart, IN). 15μm thick sections were cut on a cryostat and dried onto chrome alum-gelatin subbed slides. Sections were rehydrated, blocked using 5% normal goat serum in phosphate-buffered saline (PBS, pH 7.4) and then incubated with primary antibodies overnight at 4°C. After extensive washing with PBS, sections were incubated in the dark in goat anti-mouse or goat anti-rabbit IgG conjugated to fluorescein for 1 h at room temperature. For double-label experiments, anti-mouse IgG conjugated to rhodamine was used. In younger tissues, biotinylated secondary antibodies were sometimes used in conjunction with fluorescein-conjugated streptavidin to increase the sensitivity of the assay. After further washing, sections were mounted in 50% glycerol in PBS and coverslipped. Slides were viewed using epifluorescence and photographs taken on T-Max film. Controls were carried out to ensure no crossreactivity between mouse primary and anti-rabbit IgG, or between rabbit primary and anti-mouse IgG.

Subcellular fractionation. As previously described (Arimatsu et al., 1987; Fekete & Barnstable, 1983), retinas from neonatal and adult rats were dissected and placed into ice-cold buffered sucrose. Tissues were homogenized and centrifuged at 1000g for 10 min. The supernatant was collected and centrifuged at 40,000g for 30 min at 4°C. The supernatant was then discarded and the pellet resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The solution was then respun at 40,000g for 30 min at 4°C, and the pellet resuspended in TE buffer containing 6% sucrose.

Immunoblot. Aliquots of membrane protein were dissolved in sample buffer (2% sodium dodecyl sulfate, 5% beta-mercaptoethanol, 10% glycerol, 1% Tris-HCl pH 6.8, and 0.005% bromophenol blue), heated at 100□C for 5 min, and electrophoresed on 7.5% SDS-polyacrylamide gels (Laemmli, 1970). Proteins were then electrophoretically blotted onto nitrocellulose, blocked with 5% nonfat milk in blocking buffer (1% 1.0 M Tris, pH 8.2, 2.9% NaCl, and 0.1% Tween-20), and labelled with primary antibodies. The blot was washed extensively with blocking buffer and incubated with 1/10,000 dilution of goat anti-mouse or anti-rabbit peroxidase antibody for 1 hour at room temperature. Protein bands were visualized with ECL western blotting detection reagents (Amersham). The apparent molecular weight of each stained band was

determined by comparison with the migration of standard proteins. Protein concentrations were determined by the Lowry method (Lowry et al., 1951).

Results

Rod CNG channel expression coincides with outer segment development.

The monoclonal antibody RCNGC1-7H2 was generated against a 20-residue C-terminal peptide of the rod cGMP-gated cation channel and in adult rat retina sections labeled only the rod outer segments

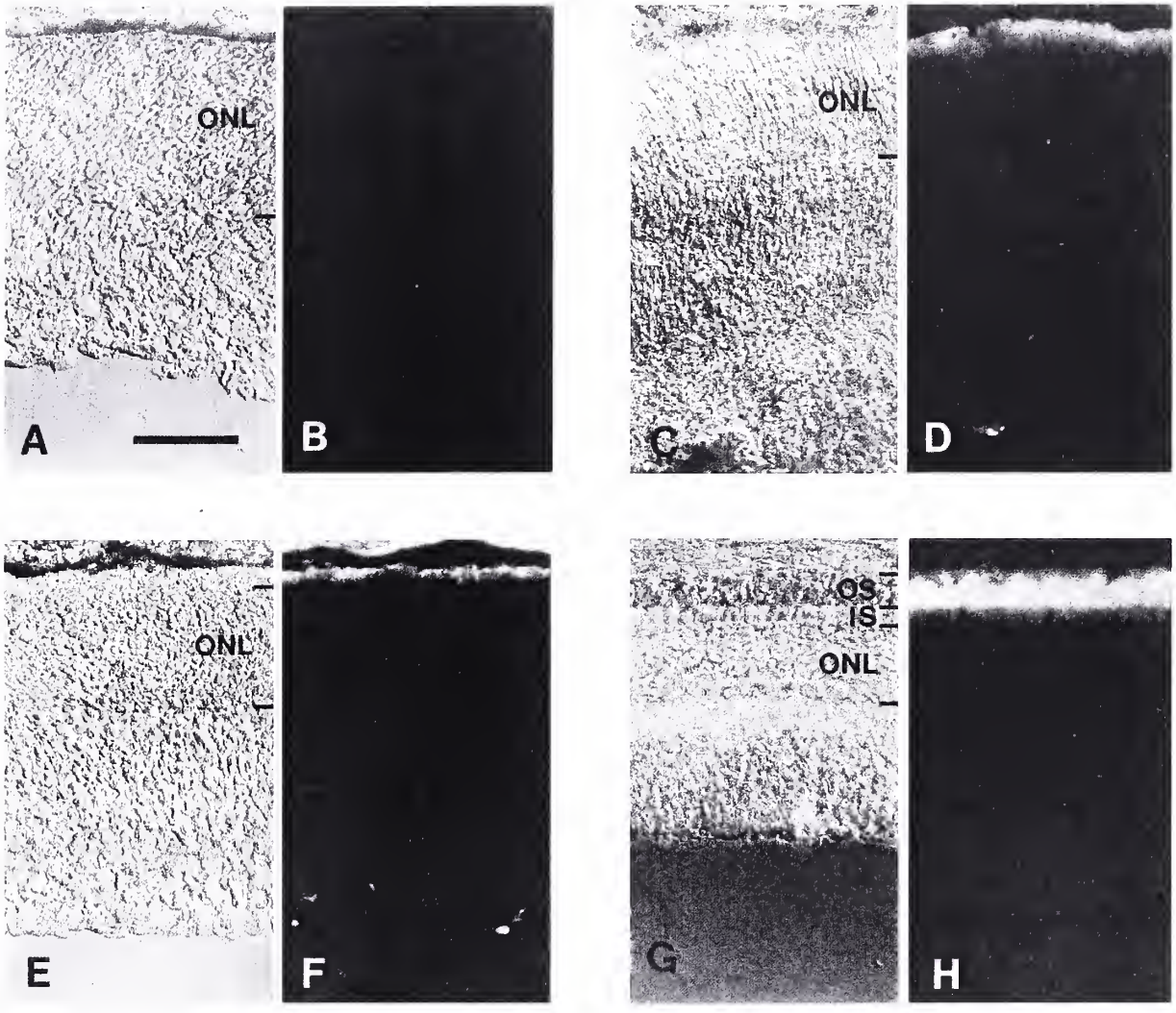

Fig. 1. Immunocytochemical development of rod cGMP-gated cation channel. A,C,E,G, phase contrast; B,D,F,H, fluorescence. (A,B) At PN5 the retina is still largely undifferentiated and no labeling by the RCNGC1-7H2 antibody is detected. (C,D) At PN7 the retina shows inner and outer segments have begun to form under the pigment epithelium and punctate labeling is seen in outer segments in central retina. (E,F) At PN10 the outer segments have greatly elongated and protrude into the subretinal space. A continuous band of intense labeling is seen on the scleral side of the neural retina. (G,H) The adult retina shows clear lamination of inner segments (IS) and outer segments (OS). An increase in the area of labeling corresponds to the elongation of outer segments with very little label in the inner segments. No label is detectable in the developing outer nuclear layer (ONL) at any age. Scale bar in A = 50 μm.

(Barnstable and Wei, 1995). Sections of PN1, PN3 and PN5 retina showed no RCNGC1-7H2 immunoreactivity (data not shown), even though by PN5 the previously undifferentiated neuroblastic zone

had begun to separate morphologically into an inner nuclear layer of rounded, differentiated cells and an outer region of more immature elongated cells and almost all rods are postmitotic by this stage (Fig 1A-B). At PN7, developing outer segments could be seen immediately adjacent to the pigment epithelium and a clear outer plexiform layer divided inner and outer retina. At this age, punctate labeling with the RCNGC1-7H2 antibody could be seen in the nascent outer segments together with a more diffuse label extending into the outer nuclear layer (Fig. 1C-D). Very faint horizontal cell labelling was also consistently observed at PN7, but not at later ages. By PN10, inner and outer segments could be seen protruding into the subretinal space, and RCNGC1-7H2 intensely labeled a continuous band of outer segments (Fig. 1E-F). Labeling of adult (>3 months) retina was similar to that of PN10 except for noticeably elongated inner and outer segments (Fig. 1G-H). Pre-incubation of RCNGC1-7H2 antibody with the C-terminal peptide immunogen removed all labeling in the outer segments of adult retina sections (Fig. 2).

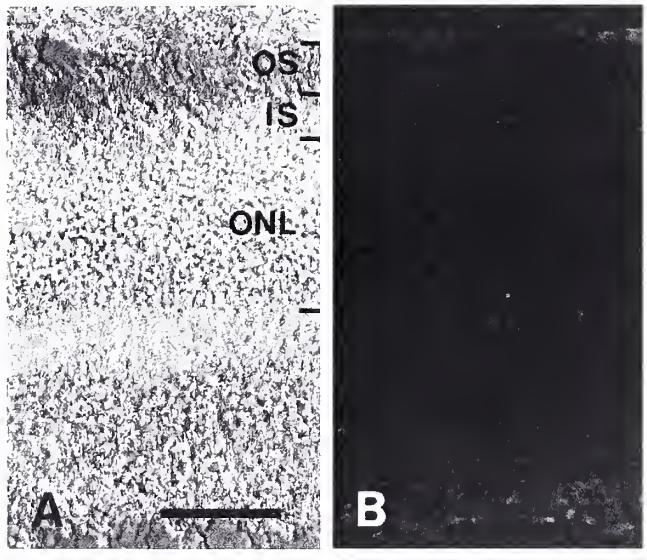

Fig. 2. Specificity of RCNGC1-7H2 for outer segments demonstrated by immunocytochemistry. (A) Phase contrast view of adult retina. (B) Fluorescence view of the same section showing that preincubation of RCNGC1-7H2 antibody with the immunising peptide completely blocks labeling of outer segments. Scale bar in A = 50 μm.

To confirm the labeling specificity we carried out immunoblots of retinal membranes on tissue of comparable ages. Like the immunocytochemistry, on these immunoblots no labeling with RCNGC1-7H2 antibody was detected at PN5 (Fig. 3A-1), but at PN7 a faint band was seen at 71 kDa (Fig. 3A-2). This band increased in intensity at PN9 (Fig. 3A-3) and was even stronger in the adult (Fig. 3A-4).

Immunoblots of adult membrane protein stained with RCNGC1-7H2 antibody preincubated with C-terminus rCNGC1 peptide also did not reveal staining at 71 kDa, despite the persistence of similar background bands (Fig. 3B-2).

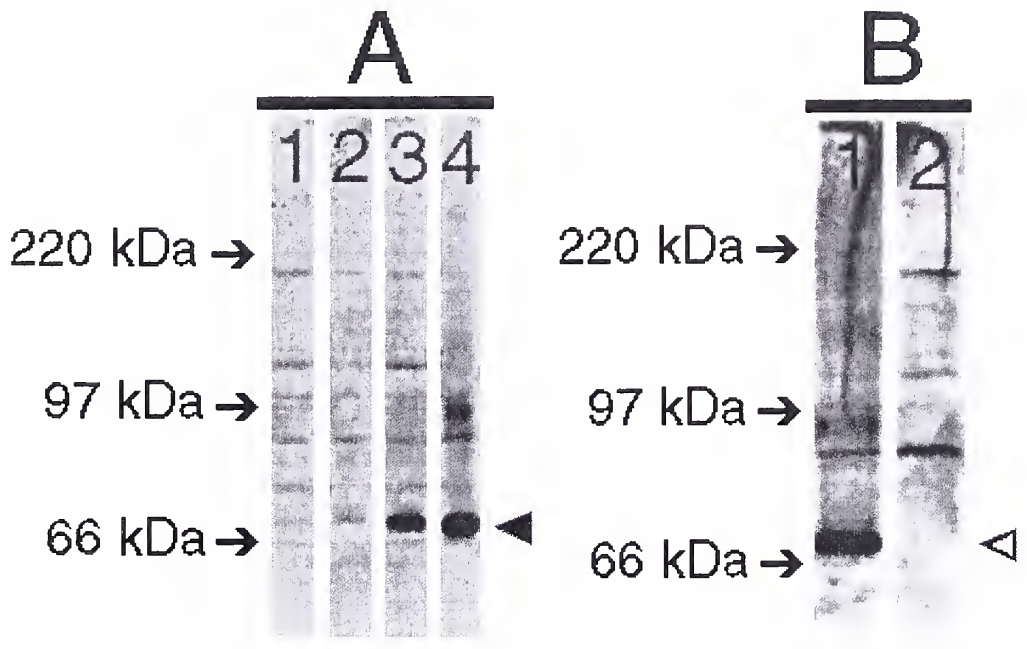

Fig. 3. Immunoblot of rat retinal membranes with RCNGC1-7H2 antibody. (A) The development of cGMP-gated cation channel protein. Labeling at 71 kDa (darkened arrowhead) is absent at PN5 (A-1), but can be faintly seen at PN7 (A-2) and increases with developmental age, PN9 (A-3) and adult (A-4). (B) Adult rat retinal membranes stained with RCNGC1-7H2 antibody (B-1) detected a strong band at 71 kDa (open arrowhead), while those stained with RCNGC1-7H2 antibody preincubated with channel C-terminus peptide (B-2) did not.

Rod outer segment-specific molecules may be under co-ordinate regulation during development.

The timing and pattern of expression of the rod CNG channel shown in Figure 1 is typical for outer segment specific molecules. For example, Figure 4 shows labeling of sections of the same retinas used for Figure 1 with antibody RET-P2 that labels the rds/peripherin molecule. Labeling is first detected at PN7 as a series of bright puncta at the outer edge of the retina together with some diffuse labeling extending partially (approximately 20 μm) into the outer portion of the outer nuclear layer (Fig. 4C-D). At PN10 and adult the labeling increased in parallel with the growth of the outer segments (Fig. 4E-H). This same pattern of labeling has also been described for other outer segment specific markers such as the rod cGMP-PDE.

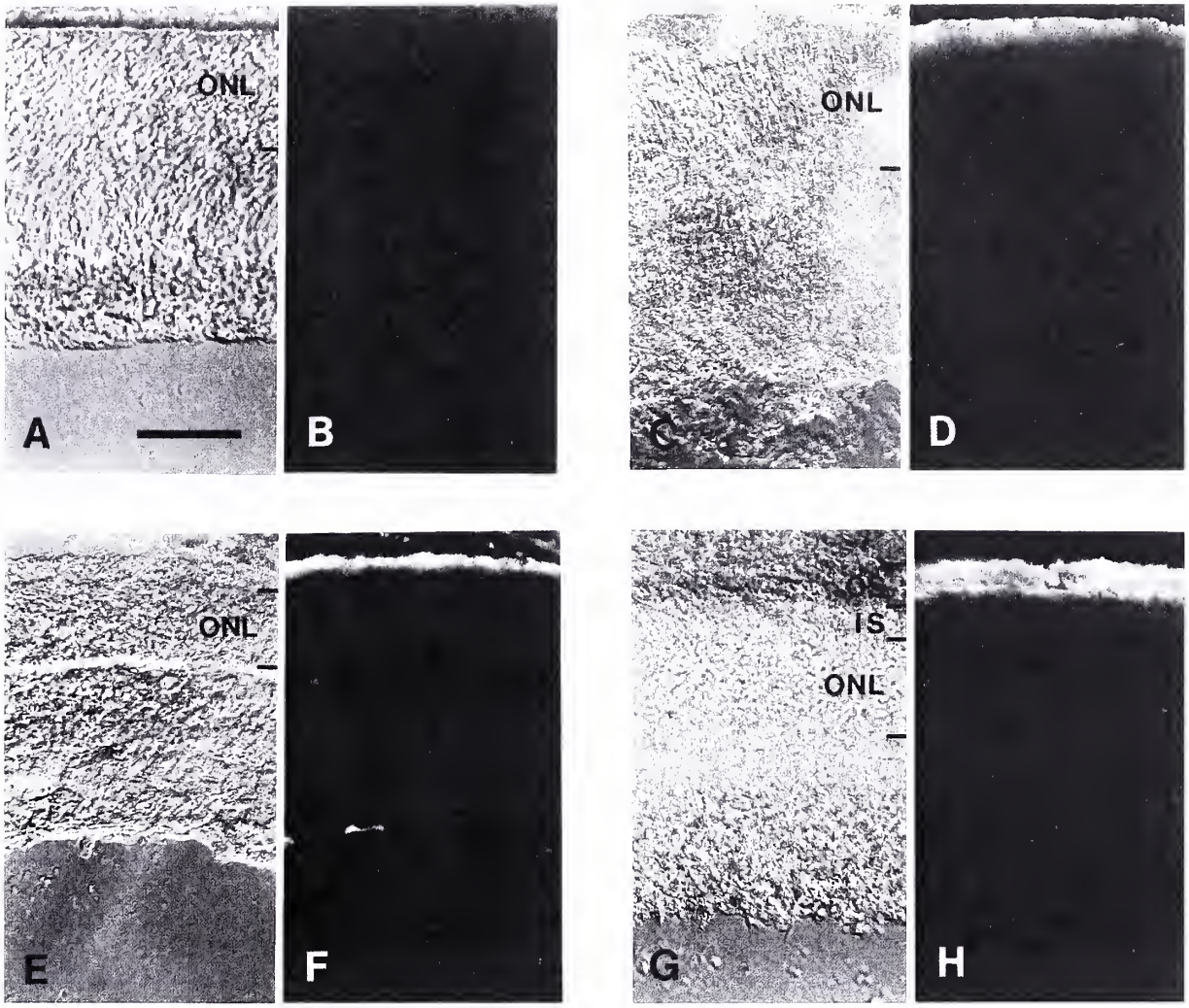

Fig. 4. Immunocytochemical labeling with RET-P2. (A) Nomarski optics of PN5 retina showing an undifferentiated outer neuroblastic zone (Onb). (B) Label is absent at PN5. (C) Nomarski optics of PN7 retina shows an outer nuclear layer (ONL) forming. (D) Punctate labelling can be seen in the outer segments (OS). (E) Nomarski optics of PN10 retina shows a well demarcated ONL. (F) Labelling is present immediately above the ONL. (G) Nomarski optics of adult retina shows an increase in OS layer thickness. (H) Labelling increases in parallel with OS growth. No label is detectable in the inner segments (IS) or ONL. Scale bar in A = 50 μm.

To determine whether the expression of these outer segment specific molecules might be regulated in concert, we carried out double-labeling experiments. As these molecules are first detected, the density of puncta representing developing outer segments is lower than at later ages, suggesting that not all rods at a given retinal location express these molecules at the same time. When the expression of the rod CNG channel (R309 rabbit antibody) and of rds/peripherin (RET-P2) were compared on the same sections, however, a complete overlap in bright puncta was observed (Fig. 5). Similar results were attained when rod CNG channel expression was compared with rod cGMP-PDE labelling (Fig. 6). This indicates that

within the resolution of this experiment, expression of these outer segment molecules occurs simultaneously in developing rods.

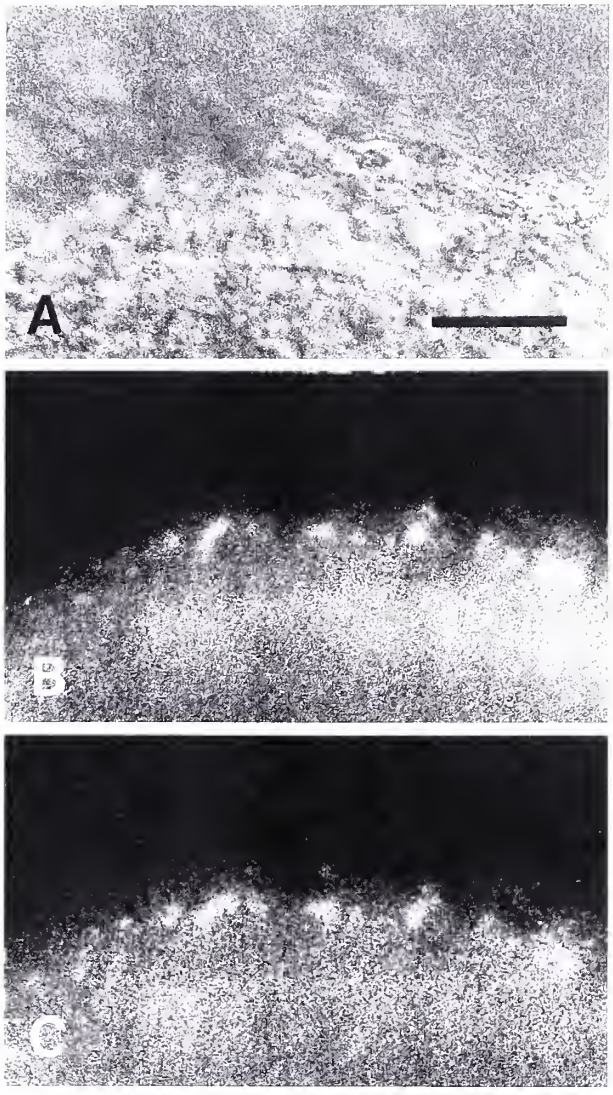

Fig. 5. Coincident expression of rod CNG channel and rds/peripherin in PN7 outer retina. (A) Phase contrast. (B,C) Identical patterns of label are seen with R309 antibody (B) and with RET-P2 antibody (C). The additional label in C over the RPE and choroid is background also seen in sections with no primary antibody. Scale bar in A = 10 μm.

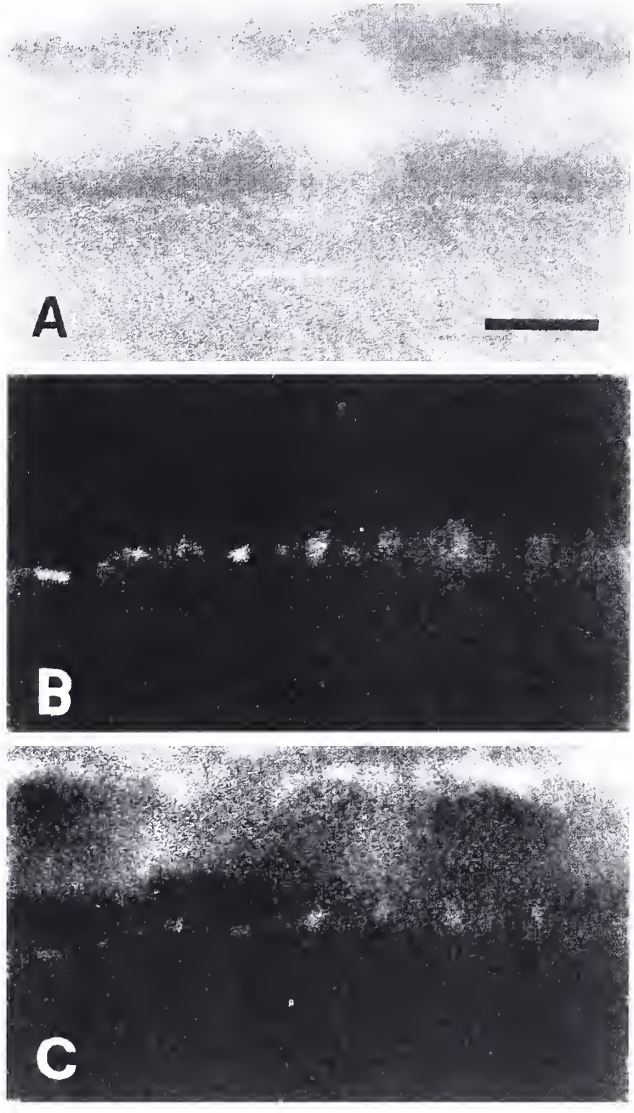

Fig. 6. Coincident expression of rod CNG channel and cGMP-PDE in PN7 outer retina. (A) Phase contrast. (B,C) Labeling of outer segments with RCNGC1-7H2 (B) and PDE-4 antibody (C) show identical patterns of labeling. Because the earliest expression is near background levels, the long exposures used also show nonspecific labeling in the outer nuclear layer. Scale bar in A = 10 μm.

Discussion

Rod photoreceptor differentiation in the rat appears to involve several distinct steps that are probably interdependent. It is well established that opsin is expressed soon after the final mitosis of rod precursors (Hicks and Barnstable, 1987b; Cepko, 1996). It is not clear whether opsin expression is induced *de novo* in a committed rod cell or is an automatic consequence of the molecular events of rod determination occuring at the time of the final mitosis of the precursor. The finding of substantial variation in the intervals between cell birth and opsin expresion in different species, and between different rods in the same species argues for some degree of induction (Watanabe and Raff, 1990; Stenkamp et al., 1997; Cepko, 1996). Interphotoreceptor Retinoid Binding Protein (IRBP) is also expressed early in rod development and may even appear before opsin (Smith et al., 1992; Timmers et al., 1993).

Outer segment formation marks a second phase of rod photoreceptor formation and involves the onset of expression of a wide range of molecules involved in the transduction cascade as well as those essential for the structural integrity of the outer segment (Colombaioni and Strettoi, 1993). We have now demonstrated by immunocytochemistry that the rod CNG channel is also expressed at the beginning of outer segment formation. In the adult, the only immunofluorescence detected was over the outer segments, as has previously been found by others (Wässle et al., 1991; Vardi et al., 1993). In other species it has been found that CNG channels can be detected electrophysiologically in cone photoreceptor terminals (Rieke and Schwartz, 1994; Savtchenko and Kramer 1997). This difference could be due to a cell type difference, a species difference or to a lack of sensitivity of the immunofluorescence assay such that low levels of expression in other compartments of the cell are not detected. At present we cannot distinguish among these possibilities.

We have previously shown that expression of rod-specific proteins in outer segments of adjacent cells does not occur synchronously (Hicks and Barnstable, 1986; Hicks et al., 1989). This has allowed us to investigate whether outer segment specific molecules appear in a random sequence or at the same time. Double-labeling studies showed complete overlap of expression of CNG channels and rds/peripherin or cGMP PDE. It will be of interest to determine whether this coincident expression is the result of coincident transcriptional activation by common *trans*-acting factors or of independent regulatory pathways linked to some common feature such as cell birthdate. It has previously been shown that an element first detected in

the proximal promoter of opsin is also found in many other rod photoreceptor genes, including some of those restricted to outer segments (Morabito et al., 1991; Kikuchi et al., 1993; Yu et al., 1995). Whether this, or other, *cis*-acting elements are responsible for the coincident expression of outer segment molecules needs further investigation.

In a previous study, we had detected low levels of rod CNG channel RNA in rat retinas several days before outer segment formation and a rapid increase as the outer segments elongated (Ahmad et al., 1990). Subsequently we found that other retinal cell types also expressed this channel (Ahmad et al., 1994; Kusaka et al., 1996). This suggests that the early low levels of expression were due to these other cell types and that the large increase represents the onset of expression in rods. This conclusion, however, depends upon the sensitivity of the immunocytochemical detection. It is possible that amounts of channel protein below the level of detection in fixed tissue are expressed earlier in the rod. This issue is of importance because it is primarily the activity of the CNG channel that regulates the membrane potential of the rod and thus can affect release of neurotransmitter. Our results suggest that the rods could become depolarized through CNG channel activation by PN7. It will be interesting to determine whether the rod guanylate cyclases are active by this time such that the concentrations of cGMP can reach levels necessary to activate the CNG channels. If so then glutamate released by the rods may influence synapse formation in both outer and inner plexiform layers which occurs over the same time period as outer segment formation (Hendrickson, 1996).

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